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SUMO (Small Ubiquitin-like Modifier) Chip for Analysis of SUMO-conjugation to a Target Protein

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Abstract

The analysis of protein interactions and modifications in a microarray format has become the focus of great interest in researchers in drug discovery, diagnostics, and cell biology, due to the advent of rapid and high-throughput biological assays requiring only a small amount of sample. Herein, we describe a chip-based analysis of sumoylation, the post-translational modification (PTM) process involving the covalent attachment of the small ubiquitin-like modifier (SUMO) family protein to a target protein in a mammalian cell. The protein was expressed using an *in vitro* translation system and immobilized directly onto a glass slide using epitope tags fused to the protein. In this paper, the microarray-based analysis technique was shown to be a useful strategy for the identification of SUMO target proteins from preexisting protein pools and proteome arrays, in a high-throughput manner.

Keywords: PTM (post translational modification), SUMO (small ubiquitin-like modifier), Protein microarray, Cell-free expression system

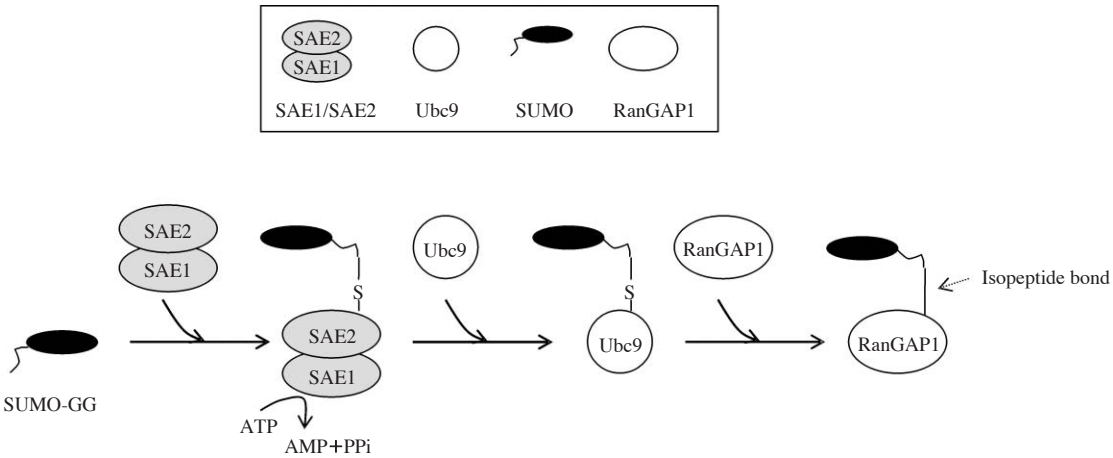
Introduction

As a novel proteomics study technique, protein chip technology has advanced significantly over the past decade, owing primarily to its utility in the high-throughput interrogation of protein interactions, modifications, and the diagnosis of proteins associated with certain diseases[1,2]. The global analysis of post-translational modifications (PTMs) of proteins has been a subject of great interest, as it plays a principal role in the maintenance of the active state, localization, and turnover of proteins, as well as in interactions with other proteins[3-5]. In recent years, protein chips have been implemented in functional activity profiling studies, allowing for a spatial assessment of the modification states of signaling proteins in cell lysates[6]. Nonetheless, chip-based analyses of PTMs have, thus far, been limited to phosphorylation studies, although approximately 300 different PTMs have already been characterized[2,3]. By way of contrast with a simple PTM process, such as protein kinase-catalyzed phosphorylation, the *in vitro* conjugation of the small ubiquitin-like modifier (SUMO) to a target protein is mediated by a three-step pathway which involves multiple enzymes, namely E1 (SUMO-activating enzyme), and E2 (SUMO-conjugating enzyme), and the mature SUMO protein[7-10].

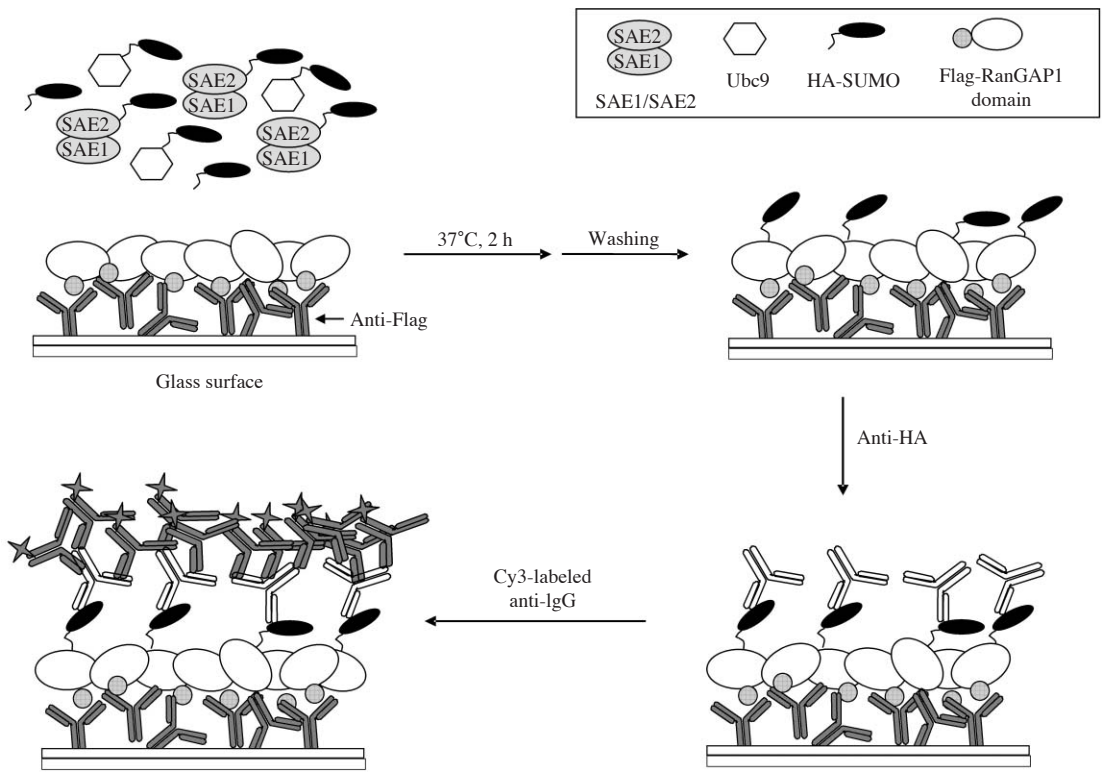
In the present study, we demonstrate the analysis of SUMO conjugation to a target protein in a microarray format. In order to achieve a chip-based sumoylation reaction, a domain protein of RanGAP1, which has previously been identified as a sumoylation target substrate[11,12], was employed as a model target substrate. As an exemplary study for parallel sumoylation analysis, we have established a chip-based sumoylation assay in a way such that a target substrate could be immobilized on a glass surface. The target substrate was then expressed via a cell-free expression system and was immobilized using epitope tags fused to the protein. The sumoylation reaction was then probed with anti-HA and dye-labeled anti-IgG antibodies (Scheme 2) under optimized conditions, as was described in our previous study[13]. The details of this experiment are provided herein.

Results and Discussion

The *in vitro* sumoylation of substrate proteins is known to require the E1 (activating enzyme) and E2 (conjugating enzyme) proteins. In a typical instance in which hetero-dimeric E1 (SAE1/SAE2) and E2 (Ubc9) proteins are employed, the mature SUMO-1 (G) is activated via a thioester linkage between the glycine residue of the SUMO and the cysteine residue of the SAE1/SAE2, and then transferred to Ubc9 in order to generate the same thioester bond. Finally, the C-terminal carboxyl group of the SUMO is attached to the ε-amino group on the lysine residue of the target substrate, which results in the stable formation of



Scheme 1. Conjugation pathway of SUMO to a target protein.



Scheme 2. Schematic procedures of chip-based fluorescence detection for sumoylation using antibody reagents on target immobilized protein microarray.

the isopeptide bond (Scheme 1).

In order to achieve a chip-based sumoylation reaction, the RanGAP1 domain and sumoylation-relevant protein components, including E1 (SAE1/SAE2), E2 (Ubc9), and HA-tagged SUMO-1 were initially expressed in *E. coli* and purified. Their biological activities were confirmed via a solution-phase sumoylation assay. The reaction conditions for *in vitro* sumoylation were identical to those described elsewhere[7,12]. Under these conditions, the RanGAP1 domain evidenced a sumoylation-shifted band for 2 h at 37°C, but no band shift was observed for the RanGAP1

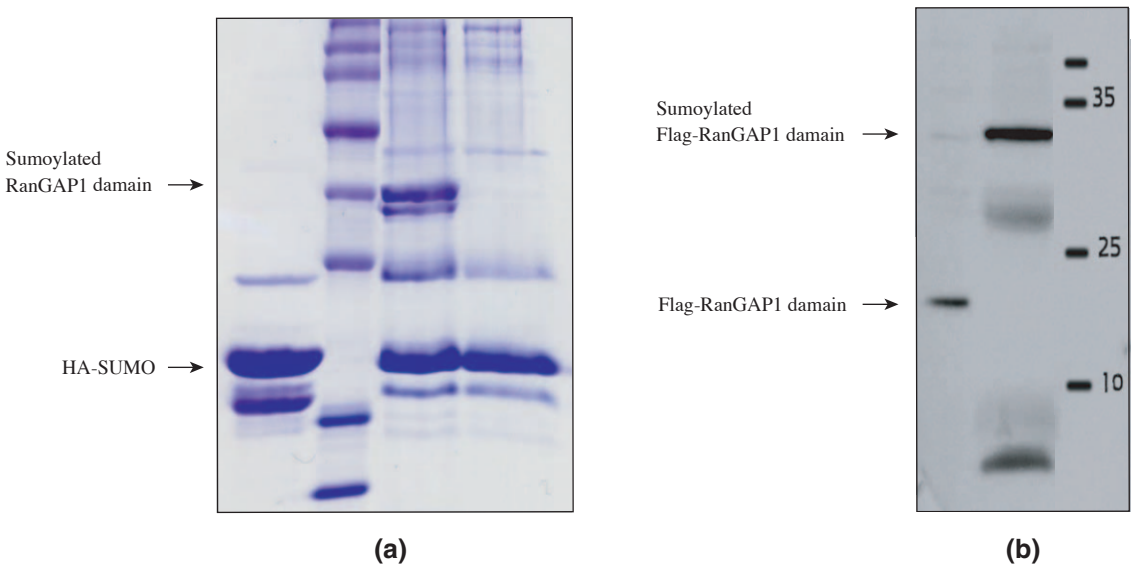


Figure 1. Solution-phase sumoylation and validation of biological activity of HA-SUMO expressed and purified *E. coli*. and flag-RanGAP1 domain expressed in a cell-free system. (a) HA-fused SUMO protein (lane 1); Molecular weight standard (lane 2); sumoylation with reaction mixture (lane 3) The reaction mixture contained protein components and additives (5 mM ATP, 5 mM $MgCl_2$, 50 mM NaCl, and 0.1 mM DTT) in 50 mM HEPES; Sumoylation in the absence of HA-SUMO (lane 4). (b) western blotting of sumoylated RanGAP1 domain. The blotted membrane was incubated with anti-flag antibody. Product translated by TNT cell-free expression system (lane 1); RanGAP1 domain sumoylated via the addition of HA-SUMO protein (lane 2). TNT rabbit reticulocyte lysates include components for sumoylation.

domain in the absence of the HA-tagged SUMO protein (Figure 1a). In order to evaluate the use of a target protein from a mammalian source with a desired efficiency, the target protein, the RanGAP1 domain, was expressed using a cell-free expression system. Using the biologically active HA-SUMO protein, we conducted a liquid sumoylation reaction in the mammalian reticulocyte lysates expressing the target protein. The expressed target protein and the SUMO-conjugated product were detected using anti-flag antibody fused to the proteins. Electrochemical luminescence (ECL) analysis indicated that the epitope-tagged RanGAP1 domain (15 kDa) was expressed abundantly in a mammalian milieu, and had almost completely shifted to a sumoylated domain (27 kDa) (Figure 1b). These results demonstrated that the relevant proteins generated by expression in *E. coli* were biologically active, and that SUMO conjugation could be detected in a mammalian cell-free system.

Unlike sumoylation in the solution phase, the on-chip sumoylation reaction requires optimization in order to proceed efficiently. In order to determine whether on-chip sumoylation can be applied to a mammalian reticulocyte lysate system, we first assessed the immobilization of a target protein expressed in a mammalian cell-free expression system. Mammalian lysates have many other proteins that are necessary for the transcription and translation of target DNA. These proteins are expected to influence the sumoylation signal as well as the background level resulting from the nonspecific adsorption of protein to a solid surface. In order to evaluate the specific immobilization of the target protein, monoclonal anti-epitope antibody fused to the expressed protein was spotted as a capture antibody. The microarray was incubated with rabbit reticulocyte lysate harboring T7 polymerase-expressing epitope-tagged protein. The captured epitope-fused protein was then detected by two approaches. One detection method involved polyclonal anti-epitope antibody, and the other employed an antibody against the target protein (Figure 2a). In cases in which antibodies are purified from different sources, access to small-sized epitope sequences becomes difficult due to the masking effects of the expressed protein, which results in a false negative signal. However, the specific immobilization of target proteins expressed in mammalian reticulocyte lysates can be detected with a target protein antibody (Figure 2b). The use of mammalian reticulocyte lysates resulted in a very low signal-to-noise (S/N) ratio.

For a reliable analysis, factors influencing the performance of the chip-based sumo-conjugation were investigated and optimized in detail. The feasibility of the protein microarray has been demonstrated

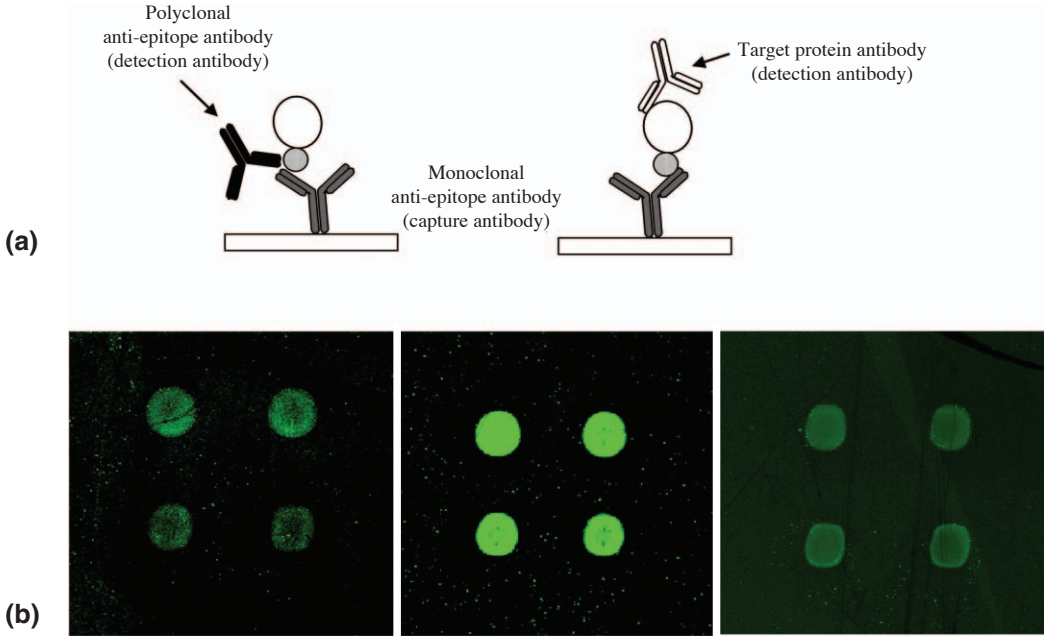


Figure 2. Strategy for immobilization of a target protein. (a) Schematic representation of on-chip immobilization with capture antibody and antibody-based detection . (b) Fluorescent images for a target protein from mammalian lysates and detection using anti-epitope antibody (left) and a target antibody (middle). Fluorescence signal of mammalian lysate after incubation without the expressed product (right). Binding was visualized using a Cy3-labeled secondary antibody.

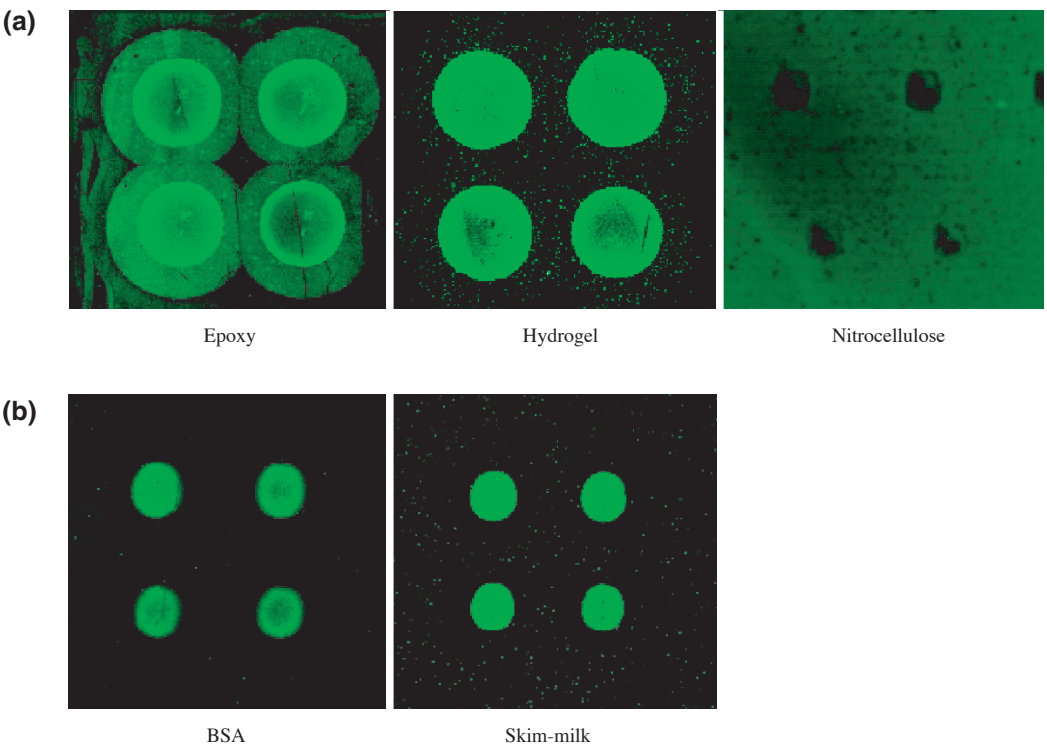


Figure 3. Optimization of reaction conditions for on-chip sumoylation. (a) Fluorescence images of spotted protein on slide glasses with various functional groups. (b) Fluorescence signal when BSA (left) and skim milk were used as blocking reagents (right) on aldehyde-modifed glass slides.

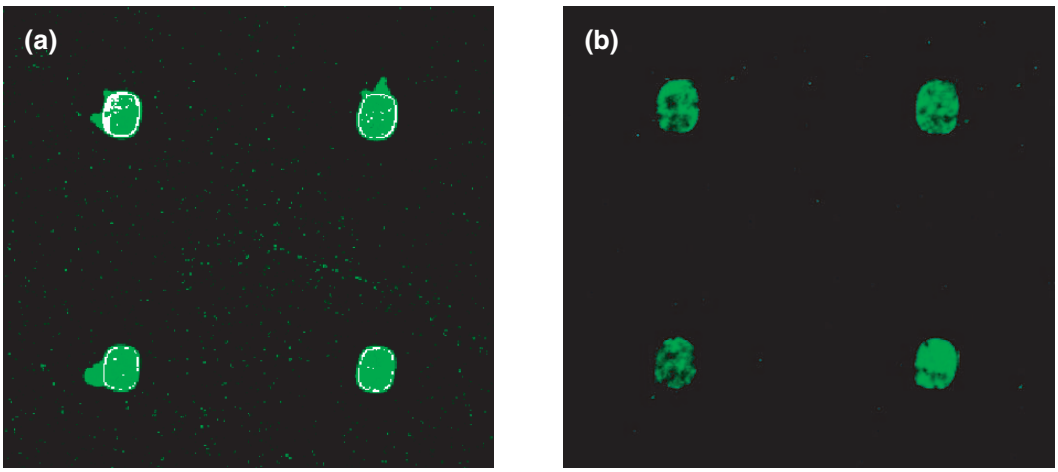


Figure 4. Fluorescence images of immobilized RanGAP1 domain and its sumo conjugation on a solid surface. (a) Expression of a target protein and its immobilization. (b) Fluorescence detection of sumo-conjugated target proteins via the reaction of anti-HA (10 μg/mL in PBST) and Cy3-labeled anti-IgG (5 μg/mL in PBST) after sumoylation.

using a variety of surfaces with different functional groups for the immobilization of capture molecules, and those include aldehyde- epoxy- group surface activation for covalent attachment, and hydrogel modification or nitrocellulose-driven polymers for non-covalent protein attachment. We evaluated the various slide glasses with regard to their capacity to immobilize target proteins expressed by mammalian lysates. In the epoxy group and hydrogel polymer-functionalized slide glasses, we detected undesirable spot morphology and larger spot sizes (395 μm) than were observed with the initial spots (150 μm). This effect may be attributable to the "spreading out" phenomenon. By way of contrast, nitrocellulose-driven polymer-coated slide glasses were observed to display a peculiar pattern when probed with Cy3-specific fluorescence intensities, and this appeared to be the result of autofluorescence (Figure 3a). In a series of tests, the highest signal-to-noise (S/N) ratio was observed using a slide glass with aldehyde groups (Figure 3b).

We then assessed the effects of blocking buffer on the efficiency of on-chip sumoylation, and examined the false signals induced by nonspecific protein adsorption. In most protein microarrays, bovine serum albumin (BSA) and skim milk are used as blocking reagents. The skim milk treatment has been observed to induce desirable fluorescence intensity (Figure 3b). Finally, the concentration of antibody reagents was determined via comparison of the observed signal from on-chip sumoylation as a function of the treated concentrations. In a previous study, we reported the optimal antibody concentrations for the specific detection of sumoylation on a glass surface[13]. As a result, we determined that the immobilization of the expressed target protein from a mammalian source could be efficiently probed on the aldehyde-modified solid surface, which had been spotted with antibody for the immobilization of a target protein. Skim milk was determined to be an effective blocking solution.

On the basis of the above results and on the results of comparisons among various types of slide glasses, it can be observed that the on-chip sumoylation of a target protein from a mammalian source was effectively conducted under the optimized reaction conditions. It is evident that the conjugation of SUMO to its bona fide target protein, locally immobilized onto a surface, can be detected using SUMO-specific antibody and secondary antibody. We initially confirmed the expression and immobilization of target proteins in the mammalian lysates using an antibody against the target protein and the Cy3-labeled secondary antibody, as described above (Figure 4a). After the sumoylation reaction, the sumoylated target protein was traced using an anti-HA antibody recognizing the epitope-fused SUMO protein and Cy3-labeled secondary antibody (Scheme 2). Via on-chip sumoylation, we determined that the on-chip sumoylation signal of a target protein generated by a cell-free expression system was relatively low, compared to that observed in cases in which the target protein was directly immobilized (Figure 4b). This may be attributed to the fact that the sumoylation efficiency of the target protein was lower than that observed in liquid phase. None-

theless, it appears obvious that SUMO conjugation to a target protein can be detected in a microarray format.

With further developments in proteome scale expression without purification steps for functional proteomics protocols, the microarray-based analysis technique described herein is anticipated to represent a novel tool for the high-throughput screening of SUMO target proteins and inhibitors.

Conclusions

In this work, we have demonstrated the fluorescence-based analysis of sumo conjugation to a target protein in a microarray format. Via the introduction of a mammalian milieu for the *in situ* expression of mammalian target proteins, a biologically active target protein was expressed and immobilized onto a glass slide. For the immobilization of a target protein, an antibody recognizing the epitope-fused target protein was spotted onto a glass surface. The qualitative evaluation of on-chip sumoylation was then conducted on the slide glass, and the factors influencing the on-chip sumoylation signal were optimized with regard to the blocking reagent, the functional groups on a slide glass, and the concentration of antibodies. As a result, the aldehyde-modified solid surface and skim milk were determined to be the most effective for the sensitive detection of sumoylation reactions. Under the optimized reaction conditions, the on-chip sumoylation of a target protein can be detected in a microarray format.

Materials and Methods

Chemical reagents. Anti-Flag antibody (developed in rabbit), Cy3-labeled anti-immunoglobulin G (anti-IgG, developed in mouse), and bovine serum albumin (BSA) were all obtained from Sigma. Monoclonal anti-HA antibody was acquired from Roche Applied Science. Cy3 and Cy5 fluorescent dyes were obtained from Amersham Biosciences and utilized in antibody labeling in accordance with the manufacturer's instructions. All other reagents employed were of the highest available quality, and were purchased from the regular source. For the buffer solutions, 50 mM HEPES (pH 7.5) was utilized as a reaction buffer for sumoylation. Phosphate-buffered saline (10 mM phosphate, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) containing 0.05% Tween 20 (PBST) and high-salt PBST containing 1 M NaCl were used. All buffer solutions were filtered through a membrane (0.2 μm cutoff) prior to use.

Plasmids. Human RanGAP1 and Ubc9 cDNAs were prepared via reverse transcription polymerase chain reaction (RT-PCR) using the total RNAs from HeLa cells as templates. The RanGAP1(418-587) was PCR-amplified and cloned into a pGEX4T-1 vector (Amersham Biosciences) with the BamHI and SalI restriction sites. The Ubc9 gene was also cloned into a pGEX4T-1 vector using the SalI and NotI restriction sites. The cDNA encoding for the RanGAP1 domain with a single mutation (K524A) was generated via PCR-directed mutagenesis and subcloned into the pET-41 vector. SUMO-1(G) cDNA was cloned into a pGEX4T-1 vector with a sequence recognizing HA. The pGEX4T-3 plasmid containing the gene encoding for the heterodimeric SUMO-activating enzyme (SAE1 /SAE2) was kindly provided by Prof. Hay (Univ. of St. Andrews, UK).

Protein expression and purification. GST-fused Ubc9 and SUMO were over-expressed in *E. coli* BL21 cells via induction with a final concentration of 0.5 mM isopropyl-D(-)-thiogalactopyranoside (IPTG). A GST-fused heterodimeric SAE1/SAE2 protein was overexpressed in *E. coli* Rosetta cells via induction with 0.1 mM IPTG at 30°C. After 3 hours of induction, the cells were harvested and resuspended in PBS containing 1 mg/mL of lysozyme. After 30 minutes of incubation on ice, the cells were lysed via sonication. The resultant lysates were centrifuged for 30 minutes at 12,000 × g, and the GST-fused proteins were purified using a column packed with glutathione -Sepharose 4B beads (Amersham Biosciences) in accordance with the manufacturer's instructions. The GST-cleaved Ubc9 and SUMO were prepared via on-column digestion with thrombin and thrombin removal, all in accordance with the manufacturer's recommended protocols . All purified proteins were stored at −70°C after desalting with 50 mM HEPES. For the *in vitro* translation of the RanGAP1 domain, we constructed a pET-41 vector system harboring the RanGAP1 (418-587) domain. The resultant plasmid was then employed as a template for *in vitro* transcription and translation using T7 RNA polymerase (Promega) under the supplier's recommended conditions. Translation reactions were conducted in TNT T7-coupled rabbit reticulocyte lysates (Promega).

On-chip sumoylation on a glass slide. The qualitative evaluation of on-chip sumoylation was conducted on slide glasses that had been modified with various functional groups, including poly-lysine (Sigma-Aldrich, St, Louis, Mo, USA), aldehyde (Telechem International, Sunnyvale, CA, USA), epoxy

(SCHOTT Nexterion, Jena, Germany), a nitrocellulose-drived polymer (FAST™ Slides, Schleicher & Schuell, Dassel, Germany), and hydrogel polymer (SCHOTT Nexterion, Jena, Germany). The antibody for the capture of the target protein was spotted onto the glass slide using a robotic arrayer (Microsys, Cartesian Technologies, Irvine, CA) equipped with a CMP3 spotting pin (Telechem International, Sunnyvale, CA). The covalent coupling of the spotted proteins was processed in a humidity-controlled chamber for 1 hour at room temperature. The antibody-immobilized surface was blocked with 5% BSA or skim milk solution for 2 hours. After 1 hour of coupling, the product from the TNT system was added to the glass slide for the immobilization of a target protein. The protein sample was then prepared in HEPES supplemented with 0.5% trehalose. After washing, the reaction with HEPES containing 0.5 M NaCl, the reaction mixture for sumoylation was added to the wells for 2 hours at 37˚C. The reaction mixture (50 mM HEPES) was composed of SAE1/SAE2, Ubc9, and SUMO proteins at various concentrations, and also contained additives of 5 mM ATP, 5 mM $MgCl_2$, 50 mM NaCl, and 0.1 mM DTT. After washing with high-salt PBST, the SUMO-conjugation was probed via serial reactions of anti-HA and Cy3-labeled secondary anti-IgG. The anti-HA solution (10 μg/mL in PBST) was initially applied to the reaction wells and incubated for 1 hour, followed by washing with high-salt PBST. The sumoylation signal was subsequently amplified via 30 minutes of reaction with Cy3-labeled anti-IgG (5 μg/mL in PBST). After thorough washing for 30 minutes with high-salt PBST, the surface was rinsed with distilled water and dried with N_2 gas prior to fluorescence analysis.

Fluorescent readout. Cy3-specific fluorescence intensities were read using a fluorescence scanner (GenePix Personal 4100A; Axon Instruments, Union City, CA, USA), and analyzed using the GenePix Pro 4.1 software provided by the manufacturer. Intensities of Cy3 collected from all spots were corrected for background intensity, and their median values were collected and analyzed for comparison of the signal-to-background ratio with the appropriate control spots.

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